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Award Number: W81XWH-12-2-0118

TITLE: Early Diagnosis and Intervention Strategies for Post-Traumatic Heterotopic Ossification in Severely Injured Extremities

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REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October-2013	Annual	30September2012 – 29September2013

4. TITLE AND SUBTITLE
Early Diagnosis and Intervention Strategies for Post-Traumatic Heterotopic Ossification in Severely Injured Extremities

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-2-0119

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This study will recruit wounded warriors with severe extremity trauma, which places them at high risk for heterotopic ossification (HO); bone formation at abnormal sites, which causes pain, limits motion and/or limits the use of a prosthetic device. There are three goals: 1) to understand the mechanisms involved in HO; 2) to define accurate and practical methods to predict where HO will develop; and 3) to define potential therapies for prevention or mitigation of HO.

15. SUBJECT TERMS
Wound healing

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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INTRODUCTION

The mechanism(s) involved in heterotopic ossification (HO) in our severely injured wounded warriors are unclear. Accurate, practical methods and assessment tools (macroscopic and cellular/molecular) need to be developed to characterize wounded tissues and predict where HO may or will develop. These tools need to provide insight into the biological wound environment and events that contribute to or elicit HO. These tools also need to provide effective methods for early diagnosis or risk assessment (prediction) so that therapies for prevention or mitigation of HO can be optimally targeted. This study seeks to contribute to advancement in each of these key areas.

The research teams at Cleveland Clinic Lerner Research Institute (CCLRI), WRNMMC and NMRC bring together robust and complementary experiences. The research team at CCLRI performs quantitative wound assessment using non-invasive imaging modalities (ultrasound), *in vitro* assay and characterization of tissue-resident connective tissue progenitors (CTPs) using image analysis of colony forming unit performance, and the teams at WRNMMC and NMRC perform Raman Spectroscopy and gene expression profiling in at-risk tissue from HO+ and HO- patients.

During Year 1, methods, protocols and SOPs were established, IRB and HRPO approvals were obtained, screening for enrollment was initiated, and insight was gained as to the biological nature of the cells and extracellular matrix environments of the wounds being studied. Communication and secure data sharing and storage resources were also established.

BODY

As of 10 June 2013, both IRB and HRPO approval has been given for the study protocol. In preparation for patient enrollment, the research team continues to refine and validate SOPs for each task of the SOW. A total of 17 SOP's have been developed and archived in the shared disk space made available to this project at WRNMMC. Patient recruitment and informed consent is performed solely by Dr. Forsberg's team at WRNMMC. As of this report, there have been no patient subjects with transfemoral amputations meeting the entrance requirements for the study. Between 10 June 2013 and the present, the incidence of transfemoral injury has fortunately been significantly less than that seen in recent years. We have begun to examine alternatives that can be executed on if the spectrum of injuries has changed, including expansion of indications to include evaluation of thigh and arm wounds ($\geq$75cm^2) with or without associated open fractures.

Some important refinements include:

Oxygen Tension Control

To allow control of oxygen tension during cell culture of CTPs from tissue samples, Dr. Davis's lab purchased a C-chamber Hypoxia Culture System (Biospherix) capable of regulating oxygen tension (0.1 to 20% range) during *ex vivo* expansion and differentiation of tissue-derived CTPs. Pilot studies were performed using human marrow-derived MSC's, amnion-derived and patient muscle-derived MSCs/CTPs progenitors (collected with patient consent; WRNNMC IRB approved protocol "Discarded Tissue Collection") to familiarize technical personnel with the equipment and Biospherix protocols. Cell samples cultured at 20%, 3% and 0.3% oxygen tension were sent to CCLRI for colony analysis.

Plating Density

Slides (~50) from de-identified tissue samples have been sent to CCLRI from NMRC to enable determination of an optimum range of plating density for colony forming unit (CFU) analysis at day 6 after plating. Densities from 10,000 to 200,000 cells/4 cm^2 well have been evaluated. CTP prevalence has been found to be high, requiring low plating densities. Two plating densities at 10,000, and 25,000 cells per chamber have been selected as preferred at this time.

Labeling Protocol

A slide label convention has been developed that defines eight (8) key variables on each slide for tracking HO+ and HO- patient samples shipped from NMRC to CCLRI.

Gene Array

A custom gene array for assessing adipogenic, chondrogenic, osteogenic, angiogenic and wound healing mRNA transcripts has been developed. A descriptive Table 1 is appended.

Histological Fixation
An SOP for fixation of cells and tissues in a manner that preserves Hyaluronic Acid (HA) was established. The NMRC processed discarded tissue samples using this SOP. These slides stained positive for HA, validating the fixation method and HA preservation during the associated shipment SOP.

Histology Analysis
A contract with Histoserv, Inc. was executed for processing human samples from NMRC. Histoserv, Inc. will process samples for histology (paraffin embedded, section, H+E and Masson's trichrome stain, and immunostain for CD3, CD14 and MPO). They will also provide three unstained sections that will be sent to CCLRI for tissue HA analysis.

Ultrasound (US)
A test of ultrasound acquired files and transfer to CCLRI was confirmed by 5 April 2013. With the assistance of Fred Gage, Dr. Jonathan Forsberg, Dr. Trevor Brown and Dr. Felipe Lisboa, Dr. Russell Fedewa completed the validation of the ultrasound data collection SOP.

Raman Spectroscopy (RS)
An SOP for *in vivo* RS of injured muscle and pre-HO tissue is in place.

Data Storage and Sharing
Key elements of data sharing across participating institutions have been addressed. Over 300 variables were considered. Sharing includes only de-identified clinical data, wound descriptors, cell harvest and plating details, and CTP quantification and characterization.

Data will be shared so that aggregate data and summary statistics may be generated on-demand by the primary investigator at each study site. A SharePoint site hosted by WRNMMC has been established for the dedicated use of this study under the Regenerative Medicine Department at NMRC and access from the WRNMMC and CCLRI has been secured. SharePoint access is password protected per user and controlled by defined user roles.

Data will be stored in Microsoft Access databases with separate data entry forms for each study site. A combined database linking shared fields will be established. Designated individuals at each location can update and curate the data they generate without restricting access to tables generated and updated by others. Version control has been established via SharePoint utilities requiring databases to be “checked out” prior to editing.

In the coming months, we plan to finalize the data tables required to capture study data at each step - from patient enrollment through final sample analyses. The interface between the individual, site-specific Access databases and the common, centralized database will require revision, as some functionality is lost when moving the development database from a local machine to the SharePoint site. We have full confidence that these remaining tasks will not hinder the progress of the study at this time and will in no way affect patient care or data quality.

KEY RESEARCH ACCOMPLISHMENTS

- IRB and HRPO approval have been obtained, enabling patient recruitment.
- All necessary methods and SOPs have been established and validated, as outlined above.
- A SharePoint site hosted by the WRNMMC and NMRC has been established for the dedicated use of this study under the Regenerative Medicine Department at NMRC and access from all sites has been secured.
- An integrated database has been designed and is ready for data entry for each step from patient enrollment to final sample analysis.

REPORTABLE OUTCOMES
The Molecular Core Laboratory (WRNMMC) created various databases for sample tracking and testing data. Suitable storage areas were identified and procured for incoming samples. SOPs were reviewed and updated

for labeling, handling and storage of samples. A SharePoint site, “Early Diagnosis for Post-Traumatic Heterotopic Ossification” hosted by the WRNNMC has been established for the dedicated use of this study under the Regenerative Medicine Department and access from NMRC and CCLRI has been secured. This site has allowed team members to share and review procedures and data entry information, and review and validate SOPs. Appended Figure 1. provides a flow chart showing each research study institution’s sample processing, data collection and sample distribution responsibilities.

CONCLUSION

The development and validation of the integrated SOPs needed for this study, shared communication resources and integrated database is non-trivial, and provides experience and methods that can be applied to future collaborative projects involving WRNMMC, NMRC and CCLRI.

Our research team has continued to work on SOP validation, and our establishment of a SharePoint site to enable capture of data from initial patient enrollment through final sample analyses has placed our team in a ready position for patient enrollment and sample processing.

Validation of these SOPs is providing preliminary evidence of the high CTP prevalence found in these wound sites, as well as evidence of hyaluronan as a significant component in the extracellular matrix within the wound healing environment.

We continue to actively screen new patients presenting at WRNMMC. Due to a potential change in the frequency and spectrum of injuries incurred going forward, and their relative risk of HO, we have begun to examine alternatives that can be executed on if the spectrum of injuries has changed.

APPENDICES

Figure 1 Flow Chart

		Development/Cell Signaling Pathways							
Symbol	**Gene Name**	**Osteo**	**Angio**	**Adipo**	**Chondro**	**Myo**	**MSC**	**Inflam**	**Housekeeping**
ACAN	aggrecan								
ADIPOQ	adiponectin, C1Q and collagen domain containing								
ADIPOR1	adiponectin receptor 1								
ALPL	alkaline phosphatase, liver/bone/kidney								
ANGPT2	angiopoietin-2								
BMP2	bone morphogenetic protein 2								
BMP4	bone morphogenetic protein 4								
BMP6	bone morphogenetic protein 6								
BSP	bone sialoprotein								
CD44	*CD44* molecule (Indian blood group)								
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha								
COL10A1	collagen, type X, alpha 1								
COL11A1	collagen, type XI, alpha 1								
COL1A1	collagen, type I, alpha 1								
COL2A1	collagen, type II, alpha 1								
COL4A3	collagen, type IV, alpha 3								
COMP	cartilage oligomeric matrix protein								
CSF3	colony stimulating factor 3 (granulocyte)								
CXCL1 (GRO)	chemokine (C-X-C motif) ligand 1								
CXCL10 (IP-10)	chemokine (C-X-C motif) ligand *10*								
CXCL12 (SDF-1)	chemokine (C-X-C motif) ligand 12								
CXCXL5 (ENA-7	chemokine (C-X-C motif) ligand 5								
ENG	endoglin								
FADP4	fatty acid binding protein 4, adipocyte								
FGF1	fibroblast growth factor 1 (acidic)								
FGF10	fibroblast growth factor 10								
FGF2	fibroblast growth factor 2 (basic)								
FLT1	fms-related tyrosine kinase 1 (VEGFR)								
GLi2	GLI family zinc finger 2								
HAS1	hyaluronan synthase 1								
HAS2	hyaluronan synthase 2								
HAT1	histone acetyltransferase 1								
HDAC1	histone deacetylase 1								
HIF1a	hypoxia inducible factor *1 , alpha* subunit								
HNF1A	HNF1 homeobox A								
IGF2	insulin-like growth factor 2								
IL-10	interleukin 10								
IL-1B	interleukin 1, beta								
IL-6	interleukin 6 (interferon, beta 2)								
IL-8/CXCL8	interleukin 8								
ITGA1	integrin, alpha 1								
ITGA2	integrin, alpha 2								
ITGAM	integrin, alpha M								
ITGAV	integrin, alpha V (vitronectin receptor)								
ITGAX	integrin, alpha X								
JAG1	jagged 1								
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)								
LEP	lLeptin								
LRP5	low density lipoprotein receptor-related protein 5								
MCP-1 (CCL2)	monocyte chemoattractant protein 1								
MIP-1a (CXCl3)	chemokine (C-C motif) ligand 3								
MMP9	matrix metallopeptidase 9								
MYOD1	myogenic differentiation 1								
NOTCH1	notch 1								
OCN	osteocalcin								
OCT4	octamer-binding transcription factor 4								
OMD	osteomodulin								
OPN	osteopontin								
PDGFA	platelet-derived growth factor alpha								
PHEX	phosphate regulating endopeptidase homolog, X-linked								
PPARG	peroxisome proliferator-activated receptor gamma								
PTCH1	patched 1								
PTK2	*PTK2* protein tyrosine kinase 2								
RHOA	ras homolog gene family, member A								
RUNX2 (Cbfa1)	runt-related transcription factor 2								
SCARB1	Scavenger receptor class B member 1								
SMO	smoothened, frizzled family receptor								
SMURF1	SMAD specific E3 ubiquitin protein ligase 1								
SMURF2	SMAD specific E3 ubiquitin protein ligase 2								
SOX2	SRY (sex determining region Y)-box 2								
SOX9	SRY (sex determining region Y)-box 9								
SP1	*Sp1* transcription factor								
Sp7 (OSX)	Sp7 transcription factor (Osterix)								
SPARC	secreted protein, acidic, cysteine-rich (*osteonectin*)								
TBX5	T-box 5								
TERT	telomerase reverse transcriptase								
TGFB1	transforming growth factor, beta 1								
TGFB3	transforming growth factor, beta 3								
TNF-a	tumor necrosis factor								
TWIST1	twist homolog 1								
VEGF-A	vascular endothelial growth factor A								
WNT5a	wingless-type MMTV integration site family, member 5A								
GUSB	glucuronidase, beta								
ACTB	Actin, beta								
B2M	beta-2-microglobulin								
GAPDH	glyceraldehyde-3-phosphate dehydrogenase								
HPRT1	hypoxanthine phosphoribosyltransferase 1								
RPL13A	ribosomal protein L13a								

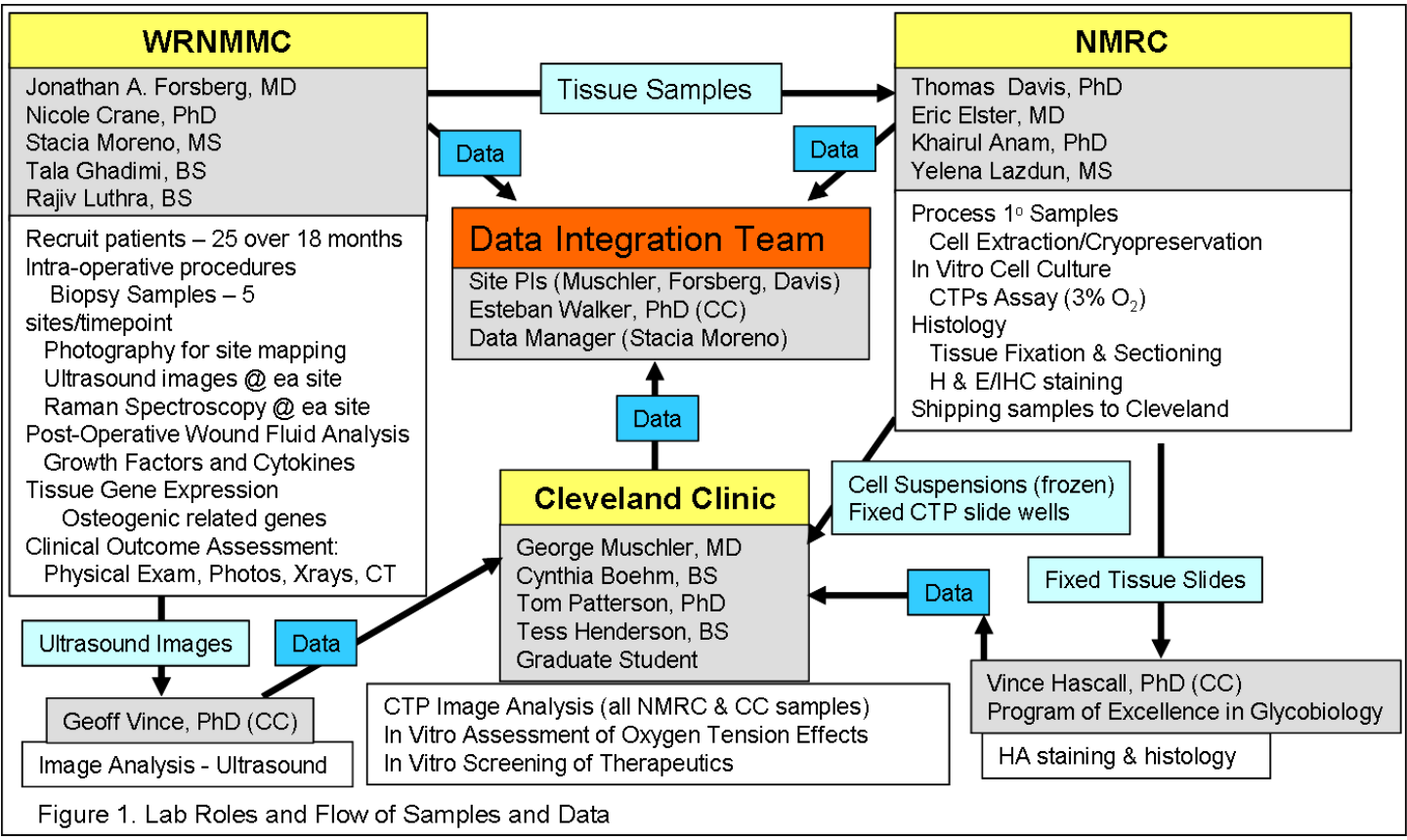

Figure 1. Lab Roles and Flow of Samples and Data